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Report

DAF-16/FOXO Regulates Transcription of *cki-1*/Cip/Kip and Repression of *lin-4* during *C. elegans* L1 Arrest

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Summary

Development is typically studied as a continuous process under laboratory conditions, but wild animals often develop in variable and stressful environments. *C. elegans* larvae hatch in a developmentally arrested state (L1 arrest) and initiate post-embryonic development only in the presence of food (*E. coli* in lab) [1–4]. In contrast to the well-studied dauer arrest, L1 arrest occurs without morphological modification, although larvae in L1 arrest are more resistant to environmental stress than developing larvae [3, 5, 6]. Consistent with its role in dauer formation and aging, we show that insulin/insulin-like growth factor (IGF) signaling regulates L1 arrest. *daf-2* insulin/IGF receptor mutants have a constitutive-L1-arrest phenotype when fed and extended survival of L1 arrest when starved. Conversely, *daf-16*/FOXO mutants have a defective-arrest phenotype, failing to arrest development and dying rapidly when starved. We show that DAF-16 is required for transcription of the cyclin-dependent kinase inhibitor *cki-1* in stem cells in response to starvation, accounting for the failure of *daf-16*/FOXO mutants to arrest cell division during L1 arrest. Other developmental events such as cell migration, cell fusion, and expression of the microRNA *lin-4*, a temporal regulator of post-embryonic development, are also observed in starved *daf-16*/FOXO mutants. These results suggest that DAF-16/FOXO promotes developmental arrest via transcriptional regulation of numerous target genes that control various aspects of development.

Results and Discussion

The insulin/IGF signaling pathway is a key regulator of dauer formation, aging, and stress resistance in *C. elegans* [7–10]. Mutations in the sole insulin/IGF receptor in *C. elegans*, *daf-2*, result in a constitutive-dauer-formation phenotype [11], a long-lived phenotype [7, 12], and resistance to oxidative [9], osmotic [8], and hypoxic [10] stress. All of these phenotypes are suppressed by mutations in the FOXO-family transcription factor *daf-16* [8–10, 13–15], indicating that the principal role of DAF-2 signaling is to antagonize DAF-16.

Because starved L1 larvae and *daf-2* mutants both arrest development and are stress resistant, we hypothesized that DAF-2 regulates L1 arrest. We determined that *daf-2* mutants have a constitutive-L1-arrest phenotype, with 10% of *daf-2(e1370)* (partial loss of function) and 70% of *daf-2(e979)* (severe loss of function) larvae arresting development after hatching in the presence of food at 25.5ºC (Figure 1A). Penetrance of constitutive L1 arrest in *daf-2* mutants increases with temperature, and wild-type worms can be forced to arrest as L1s in the presence of food at 30ºC [4] (Figure 1A). Numerous *daf-2* alleles have been reported to result in L1 arrest, the null phenotype, but it was described as L1 lethal [16], because development stops at the L1 stage at non-permissive temperatures. However, just as starvation results in stable, reversible L1 arrest, constitutively arrested *daf-2(e979)* L1s reverse arrest at 15ºC (Table 1). Furthermore, *daf-16* mutations and RNAi suppress the constitutive-L1-arrest phenotype of *daf-2* mutants (Figure 1B), indicating that DAF-2 functions principally through regulation of DAF-16 to control L1 arrest. Pharyngeal pumping is weak and sporadic in *daf-2(e979)* L1s, and it was suggested that constitutive L1 arrest may result from lack of feeding [17]. However, fluorescent beads mixed with food are easily detected in the pharynx, gut, and rectum of constitutively arrested *daf-2(e979)* L1s (Figure S1 in the Supplemental Data available online), indicating that constitutive L1 arrest does not result from lack of feeding. This observation suggests that at high temperatures the physiological state of *daf-2(e979)* mimics starvation even when fed. However, DAF-2 does not function only at high temperatures: *daf-2* mutants survive L1 arrest longer than wild-type L1s when arrested by starvation at 20ºC [4] (Figure 1C; 92% survival at 28 days for *e979* compared to 35% for the wild-type). This result demonstrates that in wild-type L1s, DAF-2 signaling activity is not completely eliminated by starvation at 20ºC and is consistent with insulin/IGF regulation of L1 arrest. Although developmental arrest is a discrete event, we believe that the state of insulin/IGF pathway activity is not all or none but instead a function of nutritional, thermal, and possibly other environmental cues with L1 arrest occurring below some critical point in pathway activity.

In contrast to *daf-2* mutants, *daf-16* mutants are defective at dauer formation [13], short lived [18], and sensitive to environmental stress [8–10]. *daf-16* null mutants are also sensitive to starvation, not surviving L1 arrest as long as wild-type L1s [4] (Figure 1C; 7% survival for *mgDf50* at 14 days compared to 86% for the wild-type). *daf-16* is epistatic to *daf-2*, indicating that starvation survival in both wild-type L1s and *daf-2* mutants requires DAF-16 function (Figure 1C). AMP-activated kinase *aak-2* and heat-shock factor *hsf-1* are known to have genetic interactions with the insulin/IGF pathway with similar mutant phenotypes to *daf-16* [19–21]. *aak-2(ok524)* and *hsf-1(sy441)* also have starvation-sensitive phenotypes (Figure 1C), lending further support to the conclusion that insulin/IGF signaling regulates L1 arrest.

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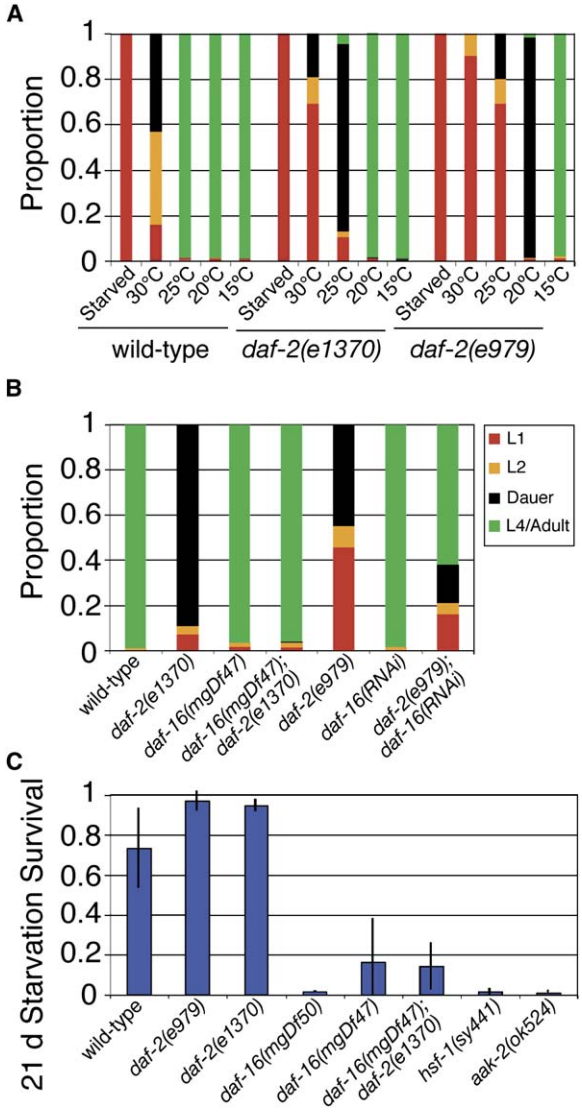

Figure 1. Insulin/IGF Pathway Regulation of L1 Arrest

(A) L1 arrest results from starvation, heat shock, and disruption of insulin/IGF receptor (DAF-2) function. The proportion of animals arrested as L1s (red), L2s (yellow), or dauers (black) or having developed to L4s or young adults (green) is plotted across a range of temperatures for the wild-type (N2) and two mutant alleles of *daf-2*. Starved worms were maintained at 20°C and scored 72 hr after eggs were prepared by hypochlorite treatment. 15°C and 20°C fed worms were also scored after 72 hr, and 25.5°C and 30°C fed worms were scored after 48 hr.

(B) *daf-16* is epistatic to *daf-2*, suppressing the constitutive-L1-arrest phenotype of *daf-2* mutants. The proportion of animals arrested as L1s, L2s, or dauers or having developed to L4s or young adults is plotted for various allelic combinations of *daf-2* and *daf-16*. Worms were cultured at 25.5°C for 48 hr after eggs were prepared by hypochlorite treatment. Bacterial-mediated RNAi was used for *daf-16(RNAi)* [38].

(C) Mutations affecting and interacting with the insulin/IGF signaling pathway affect survival of L1 arrest. The proportion of L1 larvae surviving after 21 days of starvation in S basal is plotted. The average of three independent experiments is included with error bars reflecting one standard deviation. Worms were scored as dead or alive by movement in a drop of S basal; any amount of movement, either spontaneous or in response to touch, was sufficient to be scored as alive.

Table 1. Constitutive L1 Arrest of *daf-2(e979)* Is Reversible at Permissive Temperature

	Reproductive Adult	Irreversibly Arrested or Dead	Individuals Scored
wild-type fed	100%	0%	20
wild-type starved	100%	0%	20
daf-2(e979) fed	34%	66%	100
daf-2(e979) starved	44%	56%	100
daf-2(e979) control	78%	22%	60

Eggs were hatched and larvae cultured at 25.5°C in the presence or absence of food for 2.5 days (one generation for the wild-type when fed), and then L1 larvae were picked individually to fresh plates with food and shifted to 15°C. After 16 days at 15°C, the fate of each individual L1 was scored. Because wild-type worms develop at 25.5°C when fed, developing second-generation L1s were picked as opposed to first-generation arrested L1s. For the *daf-2(e979)* control, developing L1s were picked from cultures that had been maintained continuously at 15°C. L1s that developed into dauers and crawled off the plate were scored as dead, depressing the apparent frequency of L1s that reverse arrest.

The starvation-sensitive phenotypes of *daf-16*, *hsf-1*, and *aak-2* led us to hypothesize that these mutants are arrest defective, initiating postembryonic development in the absence of food. Nomarski microscopy was therefore used to determine V lineage anatomy of starved L1s in order to infer whether postembryonic development occurs during starvation (Figure 2A). During normal development, the V lineage cells divide approximately 5 hr after hatching [22], and at least one of six V lineage cells per side had divided in 96% of *daf-16(mgDf50)* and 100% of *aak-2(ok524)* after 3 days of starvation in S basal medium [3], compared to 0% of *hsf-1(sy441)* and 15% of wild-type L1s. All six V lineage cells per side of the L1 had divided in 79% of *daf-16(mgDf50)* and 71% of *aak-2(ok524)* compared to only 3% of wild-type L1s. Ventral migration of P lineage cells and subsequent divisions were also observed in *daf-16(mgDf50)* starved L1s (data not shown; M. Fukuyama and A. Rougvie, personal communication) as well as M cell lineage divisions [at least one M cell division after 3 days starvation in 11% of *daf-16(mgDf50)* (n = 28) and 14% of *aak-2(ok524)* (n = 7) compared to 0% in the wild-type (n = 33), *hsf-1(sy441)* (n = 4), and *daf-2(e979)* (n = 10); at least one M cell division after 7 days starvation in 60% of *daf-16(mgDf50)* (n = 5) and 0% in the wild-type (n = 8); also see below]. In conclusion, *daf-16* and *aak-2* mutants initiate postembryonic development during starvation in all three lineages examined (V, M, and P), whereas the *hsf-1* mutant appears to die without developing.

The cell-cycle arrest defect observed in V lineage cells of starved *daf-16(mgDf50)* L1s can be accounted for by disrupted expression of the cyclin-dependent kinase inhibitor *cki-1* (Figures 2B–2D). *cki-1* is required for conditional cell-cycle arrest in starved L1s [2], in addition to being required for developmental cell-cycle arrest including both embryonic cell-cycle exit [23] and timing of postembryonic cell divisions [2]. As reported [2], *cki-1*::GFP expression is visible in fed and starved L1s (Figures 2B and 2C). We refer to aspects of the *cki-1*::GFP expression pattern that are common to fed and starved worms as "developmentally regulated" because they are seen during normal development in the

A

	No Divisions	Some Divisions	All 6 Divisions
3 d starvation			
wild-type	85% (33)	12% (33)	3% (33)
daf-16(mgDf50)	4% (28)	18% (28)	79% (28)
hsf-1(sy441)	100% (4)	0% (4)	0% (4)
aak-2(ok524)	0% (7)	29% (7)	71% (7)
daf-2(e979)	100% (10)	0% (10)	0% (10)
7 d starvation			
wild-type	75% (8)	25% (8)	0% (8)
daf-16(mgDf50)	0% (5)	20% (5)	80% (5)

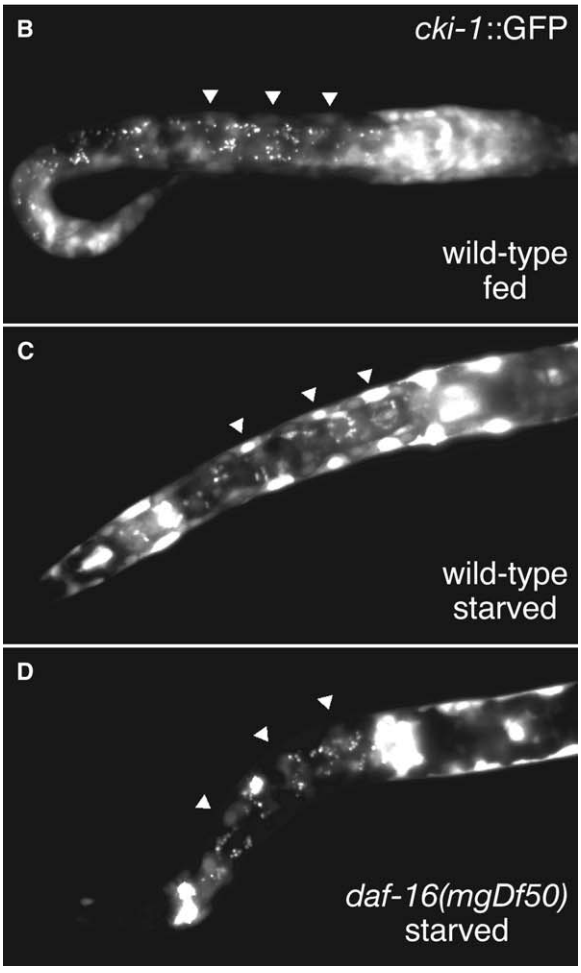

Figure 2. *daf-16* Mutants Fail to Arrest Seam-Cell Divisions during L1 Arrest and Fail to Express *cki-1* in Response to Starvation

(A) V lineage anatomy of L1 worms hatched in S basal and starved at 20ºC for either 3 or 7 days was determined by Nomarski microscopy. The number of animals scored is in parentheses; only one side of each animal was scored.

(B–D) DAF-16 is required to activate transcription of the cyclin-dependent kinase inhibitor *cki-1* in response to starvation. Worms carrying an integrated *cki-1*::GFP reporter gene were hatched in the presence (B) or absence (C and D) of *E. coli* OP50. Expression is visible mainly in neurons of the head and tail in fed wild-type L1s (B), whereas strong expression is visible in the blast cells comprising the lateral epidermal seam (V lineage) of starved wild-type L1s (C), but this upregulation is absent in starved *daf-16(mgDf50)* L1s (D). Arrowheads point to the nuclei of seam cells V1, V2, and V3 in (B)–(D) for comparison. Exposure times and image manipulations are identical for (B)–(D).

presence of food and correlate with developmental regulation of cell division. In contrast, additional "physiologically regulated" expression is visible in the V lineage cells of starved but not fed L1s (Figures 2B and 2C). These V lineage cells are called seam cells because they contribute to the lateral epidermal seam. Seam cells are stem cell-like in that they divide asymmetrically with self-renewal, one daughter destined to terminally differentiate and the other forming another multipotent seam cell [22]. In starved *daf-16(mgDf50)* L1s, physiologically regulated *cki-1*::GFP expression is absent or diminished in V lineage seam cells whereas developmentally regulated expression is undisturbed (Figure 2D), indicating that DAF-16 is required for physiologically regulated expression of *cki-1*::GFP. Because the *cki-1*::GFP reporter contains genomic regulatory sequence but no *cki-1* coding sequence, this result suggests that during starvation, DAF-16 activates *cki-1* transcription in cells that would otherwise divide, or else DAF-16 function leads to the activation of *cki-1* transcription. Mammalian FOXO family members have been shown to directly regulate transcription of the *cki-1* homolog $p27^{kip1}$ [24, 25]. However, minor residual expression of *cki-1*::GFP in seam cells of starved *daf-16(mgDf50)* L1s suggests that additional upstream pathways may contribute to physiological regulation of *cki-1*.

In addition to not arresting cell division, starved *daf-16(mgDf50)* L1s fail to arrest other aspects of development as well. For example, ventral cell migration in the P lineage occurs (data not shown; M. Fukuyama and A. Rougvie, personal communication), and the anterior daughters resulting from asymmetric division of the V lineage seam cells fuse with the dorsal hypodermal synctium hyp7 (scored with adherens-junction localized reporter *ajm-1*::AJM-1::GFP; data not shown). However, like the V lineage cell divisions, these events occur relatively early in normal L1 development. To determine how far development proceeds in starved *daf-16(mgDf50)* L1s, we focused on the development of the M cell lineage. Larvae hatch with one M cell, and it gives rise to 16 M lineage cells by the end of the L1 stage (16 hr at 20ºC) [22], but arrested wild-type L1s have only one M cell (Figure S2). An integrated *hlh-8*::GFP reporter gene [26] enables rapid identification of M lineage cells (Figure S2), allowing more animals to be scored than Nomarski microscopy. Penetrance of the M cell-division arrest defect is expected to be lower than that of the V lineage (Figure 2A) because the M cell normally divides later than the V lineage cells [22]. In this reporter-gene background, the M cell had divided at least once in 2%, 9%, and 19% of *daf-16(mgDf50)* L1s starved in S basal medium for 3, 7, and 17 days, respectively (Figure S3), indicating that penetrance of the L1-arrest-defective phenotype increases with time of starvation. However, on the basis of the number of M lineage cells per animal, the distribution of developmental stages among individuals with at least one M cell division does not change with longer periods of starvation (Figure S3). The latest developmental stage observed in a living, starved

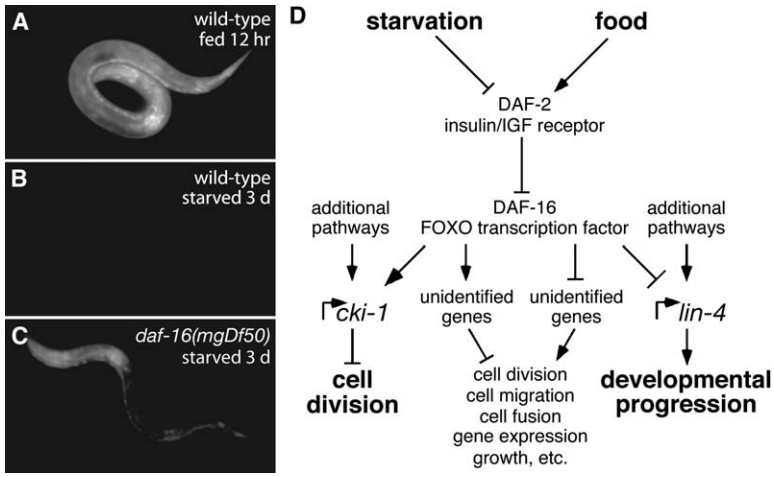

Figure 3. DAF-16 Is Required for Transcriptional Repression of the microRNA *lin-4* during L1 Arrest

(A–C)Worms carrying an integrated *lin-4*::YFP reporter were hatched in the presence (A) or absence (B and C) of *E. coli* OP50. In wild-type larvae, strong expression is visible everywhere except the four gonadal cells 12 hr after hatching with food (A), but no expression is visible 3 days after hatching without food (B). *daf-16(mgDf50)* mutants express *lin-4*::YFP even after hatching in the absence of food (C). Exposure times and image manipulations are identical for (A)–(C).

(D) A model of insulin/IGF regulation of *C. elegans* L1 arrest. DAF-2 function is modified by insulin signaling in response to nutrient availability and antagonizes DAF-16 function during regulation of L1 growth and development. Starvation causes downregulation of DAF-2 and thus upregulation of DAF-16, resulting in transcriptional activation of genes required for L1 arrest (e.g., *cki-1*) and repression of genes required for growth and development (e.g., *lin-4*). Arrows do not imply direct regulatory relationships.

daf-16(mgDf50) larvae corresponds to approximately the end of the L1 stage, when there are 16 M lineage cells. The fact that the relative frequency of the 16 M cell stage does not increase with time of starvation suggests an upper limit on how far development can proceed in the absence of food with the likelihood of lethality due to starvation increasing as development proceeds.

Multiple aspects of normal L1 somatic development occur in a roughly coordinated fashion during starvation of *daf-16(mgDf50)* L1s, suggesting that insulin/IGF signaling provides a mechanistic link in nutritional control of developmental timing. In particular, at least three somatic lineages undergo cell divisions during starvation of *daf-16* mutant L1s, and normal cell fusions and migrations are also observed. Moreover, such events are not observed in individual larvae in the absence of normally contemporaneous events (data not shown), suggesting that the relative timing of these events is intact. Accumulation of the microRNA *lin*-4, and subsequent translational repression of its targets LIN-14 and LIN-28, controls timing of events marking transition from the L1 to L2 stage, thus establishing developmental time in young larvae [27–30]. It has been hypothesized that a nutrient-sensitive pathway regulates *lin-4* accumulation because neither *lin-4* expression nor developmental events downstream of *lin-4* function are detected during L1 arrest [29]. A *lin-4*::YFP reporter gene is robustly expressed in all somatic cells of the L1 within 12 hr of hatching in the presence of food, but it is not expressed during L1 arrest (Figures 3A and 3B). Because the *lin-4*::YFP reporter gene contains only genomic regulatory sequence fused to the YFP coding sequence, this result demonstrates that *lin-4* transcription is repressed by starvation and/or activated by nutrition, as opposed to a posttranscriptional regulatory mechanism. Consistent with other aspects of the L1-arrest-defective phenotype of *daf-16(mgDf50)*, *lin-4*::YFP is expressed in starved *daf-16(mgDf50)* L1s (Figure 3C). However, the penetrance of *lin-4*::YFP expression is relatively low, visible in only 3% and 8% of larvae after 3 and 7 days of starvation, respectively [0% in wild-type and *hsf-1(sy441)* L1s]. It is intriguing to speculate that nutritional control of developmental timing could be mediated by direct transcriptional regulation of *lin-4* by DAF-16. However, given the low penetrance of the *lin-4*::YFP-expression phenotype together with the presumption that DAF-16 function likely results in global repression of gene expression during starvation, the simplest interpretation is that DAF-16 represses *lin-4* transcription indirectly. Thus, the low penetrance of the *lin-4*::YFP-expression phenotype in starved *daf-16(mgDf50)* L1s likely reflects the proportion of individuals that develop to the relatively late period in the L1 stage when *lin-4*::YFP expression becomes visible. Alternatively, DAF-16 could regulate *lin-4* directly, but initial expression levels could be too low for detection by reporter gene.

Conclusions

We have identified insulin/IGF signaling as a critical regulator of L1 arrest. Consistent with the constitutive-dauer-formation, long-lived, and stress-resistant phenotypes of *daf-2* mutants, severe loss of *daf-2* function results in reversible, constitutive L1 arrest [16] (Figures 1A and 1B; Table 1). *daf-2* mutants also survive starvation after hatching (L1 arrest) longer than wild-type L1s, and *daf-16*, *aak-2*, and *hsf-1* mutants are sensitive to starvation, dying faster than wild-type L1s in the absence of food [4] (Figure 1C). *daf-16* and *aak-2* mutants are L1 arrest defective, initiating postembryonic somatic development in the absence of food (Figure 2A; Figure S3).

Multiple aspects of normal development occur in starved *daf-16* mutants (e.g., cell division in V, M, and P lineages; P cell migration; hyp7 fusion; and expression of the microRNA *lin-4*, a primary regulator of developmental timing) (Figure 2A; Figure 3C; Figure S3), suggesting that DAF-16 promotes developmental arrest by regulating transcription of genes that control different aspects of development (Figure 3D). As one example

of such regulation, we show that DAF-16 is required for activation of *cki-1* transcription in response to starvation (Figures 2B–2D), resulting in cell-cycle arrest [2]. *Drosophila* FOXO activates 4E-BP expression in response to starvation, thereby globally downregulating translation and implementing a metabolic brake [31, 32]. A similar mechanism likely exists in *C. elegans*, as well as other analogous mechanisms targeting the transcription machinery. Such global regulation of gene expression by DAF-16 could account for indirect repression of a variety of developmental events during L1 arrest, including expression of *lin-4*.

The results presented here implicate insulin/IGF signaling in the nutritional control of development during the first *C. elegans* larval stage, although additional DAF-16-independent pathways may also participate. The identity and source of insulin/insulin-like ligands that regulate L1 arrest in response to environmental stress is unknown. There are 38 predicted insulin-like peptide-encoding genes in *C. elegans*, and overexpression of some of them results in constitutive L1 arrest [33]. Although nutrient levels can be sensed cell autonomously, it is likely that signaling in specific tissues (e.g., neurons and intestine) regulates L1 arrest, as is the case with longevity [34, 35], and as evidenced by the fact that mosaic arrest is not observed, even under intermediate conditions resulting in variable arrest stages (data not shown). In addition to affecting dauer formation, mutations disrupting chemosensory-neuron function enhance the constitutive-L1-arrest phenotype of *daf-2* mutants [36], consistent with neuroendocrine regulation of L1 arrest. Insulin/IGF signaling may regulate developmental arrest in response to other forms of environmental stress as well. Nutrient-sensitive insulin-like signaling also controls stem cell division in *Drosophila* [37], and mammalian FOXO family members mediate cell-cycle regulation via transcriptional activation of $p27^{kip1}$ [24, 25]. These observations suggest that insulin/IGF signaling regulates cyclin-dependent kinase inhibitors in other animals as in *C. elegans* and that such a conserved mechanism for physiological control of development may be modified during cancer progression.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and three figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/16/8/780/DC1/.

Acknowledgments

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